BBA - General Subjects 1862 (2018) 377–384

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

# Cell milieu significantly affects the fate of AApoAI amyloidogenic variants: predestination or serendipity?

Rosa Gaglione[a,1], Giovanni Smaldone[b,1], Rocco Di Girolamo[a], Renata Piccoli[a,c], Emilia Pedone[d,e,*], Angela Arciello[a,c,**]

[a] Department of Chemical Sciences, University of Naples Federico II, 80126 Naples, Italy
[b] IRCCS SDN, Via E. Gianturco 113, 80143 Naples, Italy
[c] Istituto Nazionale di Biostrutture e Biosistemi (INBB), Italy
[d] Istituto di Biostrutture e Bioimmagini, CNR, Naples, Italy
[e] Research Centre on Bioactive Peptides (CIRPeB), University of Naples Federico II, Via Mezzocannone 16, 80134 Naples, Italy

ARTICLE INFO

*Keywords:*
Apolipoprotein A-I
Conformational diseases
Amyloidosis
Fibrillogenesis

ABSTRACT

*Background:* Specific apolipoprotein A-I variants are associated to severe hereditary amyloidoses. The organ distribution of AApoAI amyloidosis seems to depend on the position of the mutation, since mutations in residues from 1 to 75 are mainly associated to hepatic and renal amyloidosis, while mutations in residues from 173 to 178 are mostly responsible for cardiac, laryngeal, and cutaneous amyloidosis. Molecular bases of this tissue specificity are still poorly understood, but it is increasingly emerging that protein destabilization induced by amyloidogenic mutations is neither necessary nor sufficient for amyloidosis development.

*Methods:* By using a multidisciplinary approach, including circular dichroism, dynamic light scattering, spectrofluorometric and atomic force microscopy analyses, the effect of target cells on the conformation and fibrillogenic pathway of the two AApoAI amyloidogenic variants AApoAI$^{L75P}$ and AApoAI$^{L174S}$ has been monitored.

*Results:* Our data show that specific cell milieus selectively affect conformation, aggregation propensity and fibrillogenesis of the two AApoAI amyloidogenic variants.

*Conclusions:* An intriguing picture emerged indicating that defined cell contexts selectively induce fibrillogenesis of specific AApoAI variants.

*General significance:* An innovative methodological approach, based on the use of whole intact cells to monitor the effects of cell context on AApoAI variants fibrillogenic pathway, has been set up.

## 1. Introduction

### 1.1. Familial systemic amyloidoses associated to amyloidogenic variants of apoA-I

Protein misfolding, aggregation and consequent formation of insoluble fibers are hallmarks of amyloidoses. At the present, > 40 polypeptides have been found to be implicated in amyloidoses. Among these, apolipoprotein (apo) family is over-represented [1]. This might be due to these proteins high hydrophobicity and low structural stability, which are necessary for their biological activity, but also promote β-aggregation [1,2]. Human mature apolipoprotein A-I (apoA-I) is a 243 amino acid long protein expressed mainly in the liver and the small intestine [3]. It is firstly synthetized as a 267-residue pre-pro-protein [4]. Upon cleavage of the signal peptide, it is secreted as pro-apoA-I into the plasma, where it is finally processed into its mature form [2]. ApoA-I is the major structural and functional protein on high density lipoproteins (HDL), that are committed to the removal of excess cholesterol from peripheral tissues to the liver, thus protecting the organism against atherosclerosis [5,6]. The majority of plasma apoA-I (about 95%) circulates in a stable α-helical conformation bound to HDL [7–9], whereas a small portion of it (about 5%) forms a labile lipid-

*Abbreviations:* ApoA-I, apolipoprotein A-I; HDL, high density lipoproteins; AApoAI, apolipoprotein A-I amyloidogenic variant; AApoAI$^{L75P}$, AApoAI variant with leucine to proline substitution at position 75; AApoAI$^{L174S}$, AApoAI variant with leucine to serine substitution at position 174; ThT, Thioflavin T; CD, circular dichroism; AFM, atomic-force microscopy; DLS, dynamic light scattering

* Correspondence to: E. Pedone, Istituto di Biostrutture e Bioimmagini, CNR, Via Mezzocannone 16, 80134 Naples, Italy.
** Correspondence to: A. Arciello, Department of Chemical Sciences, University of Naples Federico II, Via Cintia 4, 80126 Naples, Italy.
*E-mail addresses:* empedone@unina.it (E. Pedone), anarciel@unina.it (A. Arciello).
[1] These authors equally contributed to the paper.

https://doi.org/10.1016/j.bbagen.2017.11.018
Received 21 July 2017; Received in revised form 17 November 2017; Accepted 21 November 2017
Available online 23 November 2017
0304-4165/ 

poor/lipid-free monomer [10], the so called "free" apoA-I. This is a transient species that may encounter several fates: i) it rapidly binds to lipoproteins; ii) it interacts with plasma membrane and is involved in nascent HDL biogenesis; iii) it can be degraded by so-far unidentified proteases; iv) it can misfold and deposit as fibrils. Familial systemic amyloidoses associated to amyloidogenic variants of apoA-I (AApoAI) are autosomal dominant genetic diseases prototypically associated to the gain of a pathological function [3]. They are characterized by the deposition of N-terminal fragments of apoA-I variants, 83–93 residue long, as fibrils in vital organs and tissues, with consequent organ failure [3,11]. The only current treatment is end-stage organ transplant. Most of the known mutations seem to favor the proteolytic cleavage of apoA-I at a region comprising residues 83–100 [12], downstream to a putative β-breaking motif (EKETEG) encompassing residues 76–78 [2]. *In vitro* studies have demonstrated the high propensity of 1–93 fragment of apoA-I, the main constituent of apoA-I fibrillar deposits located in the heart [3], to form amyloid fibrils in acidic conditions [13–15], and to induce conformational disorder in an ordered lipid bilayer [16]. However, in the case of acquired amyloidosis, full-length wild-type apoA-I deposits as fibrils in arterial plaques [14,17–19], thus contributing to atherogenesis [20].

### 1.2. Effects of amyloidogenic mutations on AApoA-I folding

AApoAI mutations have been mapped on the atomic structure of lipid-free human Δ(185–243) apoA-I, a C-terminally truncated protein that contains all ~20 known AApoAI mutation sites [21]. This led to hypothesize that amyloidogenic mutations destabilize the N-terminal helix bundle and weaken protein–lipid interactions, thus favoring apoA-I misfolding [21]. Bioinformatics analyses allowed the identification of four hydrophobic segments, corresponding to residues 14–22, 53–58, 69–72 and 227–232, with high propensity to aggregate *via* the N-terminal region [22–25]. They are normally protected from misfolding by their native α-helical structure in free or lipid-bound apoA-I [2]. In the case of mutations or other modifications, a reduced structural protection of these segments would promote apoA-I misfolding. Indeed, it has been proposed that a perturbed packing of these amyloid "hot spots" might cause their conversion from the native α-helical conformation into a parallel intermolecular β-zipper that propagates from the N- to the C-terminus, thus determining β-amyloid protein aggregation of either the full-length protein (in acquired amyloidosis) or of its N-terminal 9–11 kDa fragment released by a proteolytic event [22]. In this scenario, protein misfolding would precede AApoAI variants proteolytic cleavage, although it cannot be excluded that proteolysis could precede or occur in parallel with protein aggregation [2]. AApoAI amyloidogenic mutations have been defined as "inside" mutations when they are located within the fibrillogenic N-terminal segment, and "outside" mutations when they are located outside this region, mainly within residues 170–178 [26]. Interestingly, both types of mutations are located in the four helix bundle in protein 3D structure. Recently, a biophysical characterization of AApoA-I naturally occurring variants indicated that the large global destabilization of free or lipid-bound apoA-I is neither necessary nor sufficient for amyloidosis [22]. Indeed, following mutations or post-translational modifications, amyloid hot spots are exposed to protein environment, and this might determine a shift of the balance from physiological protein clearance to aberrant aggregation [27]. Nevertheless, several additional factors might play a key role in AApoAI associated amyloidoses.

### 1.3. Tissue specificity of AApoAI associated amyloidoses

Intriguingly, the organ distribution and clinical presentation of AApoAI amyloidosis may depend on the position of the mutation [28]. Indeed, patients with alterations in amino acids from 1 to 75 mostly develop hepatic and renal amyloidosis, while carriers of mutations in residues from 173 to 178 mainly suffer from cardiac, laryngeal, and cutaneous amyloidosis [28]. Here, we analyze for the first time the effects of the cell context on the fibrillogenic pathway of two AApoAI amyloidogenic variants by using a multidisciplinary approach. In particular, we focused our attention on L75P and L174S AApoAI amyloidogenic variants, the former representing an example of "inside" mutation and the latter an example of "outside" mutation. AApoAI variant with leucine to proline substitution at position 75 ($AApoAI^{L75P}$) is associated with a hereditary systemic amyloidosis characterized by preferential accumulation of fibrils in kidneys and liver [1], whereas the variant with leucine to serine substitution at position 174 ($AApoAI^{L174S}$) is responsible for a predominant accumulation of fibrils in heart, skin, testes and larynx [2]. In order to mimic amyloidogenic proteins natural targets, human hepatocytes or rat cardiomyoblasts were here incubated with wild-type protein or with the two AApoAI amyloidogenic variants. Upon incubation, the effects of specific cell milieus on the fibrillogenic pathway of AApoAI variants were evaluated. Interestingly, from our data, a picture emerged indicating that different cell contexts play a key role in selectively inducing fibrillogenesis of specific AApoAI variants.

## 2. Materials and methods

### 2.1. Materials

All reagents were purchased from Sigma-Aldrich, Milan, Italy, unless differently specified. Protease inhibitors cocktail was from Roche Applied Science, Mannheim, Germany. Rat embryos heart myoblasts H9c2 and human hepatic carcinoma HepG2 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM, supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, Logan, UT, USA) and antibiotics, in a 5% $CO_2$ humidified atmosphere at 37 °C. The growth medium of H9c2 cells was implemented with 2 mM L-glutamine and 2 mM sodium pyruvate.

### 2.2. Production of recombinant proteins

A bacterial expression system consisting of apoA-I expressing pET20 plasmid in *Escherichia coli* strain BL21(DE3) pLysS (Invitrogen, Waltham, Massachusetts, USA) was used to prepare proteins, as previously described [26]. Proteins were purified as previously described [26], and stored at − 20 °C until use. As previously reported, for all the experiments, His-tag containing proteins were used [26].

### 2.3. CD spectroscopy

CD spectra of apoA-I and its amyloidogenic variants were recorded with a J-810 spectropolarimeter equipped with a Peltier temperature control system (Model PTC-423-S, Jasco Europe, Cremella, LC, Italy). Far-ultraviolet (Far-UV) measurements (198–260 nm) were carried out in 20 mM Tris-HCl pH 7.4 at 20 °C by using a 0.1 cm optical path length cell. Spectra were recorded with a time constant of 4 s, a 1 nm bandwidth, and a scan rate of 20 nm $min^{-1}$. The value of molar ellipticity is referred to molar ellipticity *per* mean residue (Θ) expressed in deg. $cm^2$ $dmol^{-1}$. It was calculated using the following equation: $[\Theta] = [\Theta]_{obs}$ $MRW/10lC$, where $[\Theta]_{obs}$ is the ellipticity measured in degrees (mdeg), MRW is the mean residue molecular weight (molecular weight of the protein divided by the number of amino acid residues), C is the protein concentration (g $mL^{-1}$) and *l* is the optical path length of the cell (cm).

Prior to analyses, proteins (10 μM) were pre-incubated with H9c2 ($1.25 \times 10^5$) or HepG2 ($3.75 \times 10^5$) cells [29,30] in 20 mM Tris-HCl pH 7.4 at 20 °C. Raw spectra were corrected for cell contribution. Three acquisitions for each spectrum were recorded. For each sample, three independent experiments were performed by using different protein preparations and independently grown cells.